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(54) Title: TOLEROGENIC IMMUNOGLOBULIN-PROTEIN CONJUGATES

(57) Abstract

A tolerogenic conjugate for inhibiting an immune response in a mammal to a protein, the conjugate comprising the protein, or a fragment or peptide thereof, covalently linked to a soluble isologous immunoglobulin carrier molecule.

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TOLEROGENIC IMMUNOGLOBULIN-PROTEIN CONJUGATES

This invention was made in the course of work performed under grants or awards from the National Institutes of Health. The U.S. government has rights in the invention.

Background of the Invention

This application is a continuation-in-part of U.S.S.N. 280,324, filed December 6, 1988.

This invention relates to the inhibition of unwanted immune responses, to proteins which are either foreign (i.e., heterologous) or native (i.e., isologous). In the case of such responses to native proteins, the immune system reacts to a natural body protein as if it were a foreign antigen, that causes what are known as the autoimmune diseases. Examples of autoimmune diseases are Systemic Lupus Erythromatosus (SLE) and Myasthenia Gravis (MG).

SLE is a disease in which the patient produces auto-antibody to a wide variety of antigens, including, probably most significantly, antibody to the patient's own DNA. SLE has been treated *in vitro* using a tolerogenic conjugate of an oligonucleotide covalently linked to an isologous IgG carrier. The conjugate was shown to inhibit the production of anti-DNA antibodies by cultured lymphoid cells from SLE patients. Because of this ability a conjugate having the ability to inhibit the immune response of a mammal, particularly a human, to an immunogen, the conjugate can be said to be tolerogenic. (Borel et al., U.S. Patent No. 4,650,675, hereby incorporated by reference).

Non-immunogenic carrier molecules other than

immunoglobulin that have been used to induce tolerance are D-GL (D-aminoglutamic acid L)(Katz et al., 1971, J. Exper. Med. 134:201), carboxmethyl cellulose (Diner et al., 1979, J. Immunol. 122:1986), and polyethylene glycol (Wilkinson et al., 1987, J. Immunol. 139:326).

Golan et al. (1971, J. Exptl. Med. 137, 1046) says that "the induction of tolerance . . . may be strongly influenced by the carrier moiety", and that "among the various carriers tested, IgG was the most tolerogenic."

Summary of the Invention

The invention features a tolerogenic conjugate for inhibiting a mammal's immune response to a protein, the conjugate is composed of the protein, or a fragment of peptide thereof (preferably an immunogenic fragment or peptide, encompassing an immunodominant portion of the protein) covalently linked to a soluble isologous (i.e., human) immunoglobulin (preferably IgG) carrier molecule. The protein can be a foreign protein, e.g., a therapeutic monoclonal antibody, or an isologous protein which acts as a causative agent in an autoimmune disease such as SLE. (As used herein, "causative protein" means the protein which causes symptom(s) of the unwanted immune response and to which the destructive antibodies are made; "protein fragment" means a proteolytic portion of a protein; and "peptide thereof" means a synthetic portion of the protein.)

The conjugates of the invention, when administered to a patient, is capable of inducing tolerance in a patient suffering from an unwanted immune response. The conjugate is administered to the patient by conventional methods, e.g., intraveneously admixed with a pharmaceutically acceptable carrier such as saline. The immunoglobulin portion functions not only as a carrier

molecule for the protein, peptide or fragment, but also, it is believed, helps to induce tolerance in some circumstances.

As is mentioned above, the causative protein, protein fragment, or peptide can correspond to the entire protein molecule, or to an immunodominant portion of the protein; an example of immunodominant region of a causative protein is amino acids 172-204 of the a subunit of the acetylcholine receptor for the autoimmune disease Myasthenia Gravis. Other causative proteins which can be used to make tolerogenic conjugates of the invention include P protein, associated with SLE, Factor VIII Light chain, associated with an autoimmune response to the blood coagulation Factor VIII, and insulin, associated with insulin-dependent diabetes.

The covalent crosslinking reagent used to form the conjugate is preferably a homobifunctional reagent, e.g., disuccinimidyl suberate (DSS).

Tolerogenic conjugates of the invention can be used to treat any autoimmune diseases in which a protein or peptide acts as a causative agent by generating an unwanted immune response. Examples are the autoimmunity that develops from repeated treatment of hemophiliac patients with Factor VIII; the central nervous system manifestations of SLE, pemphigus vulgaris, or myasthenia gravis; and from diabetes.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Preferred Embodiments

We turn now to a description of preferred embodiments of the invention, after first briefly describing the drawings.

Drawings

Fig. 1 is a graph showing the antibody response to Factor VIII Light chain in two Balb/c mice preimmunized to Factor VIII light chain.

Fig. 2 is a graph showing the antibody response to Factor VIII Light chain in adult mice preimmunized to Factor VIII.

Fig. 3 is a graph showing a T cell proliferation assay to cytochrome C in B10.A mice.

Fig. 4 is a graph showing the long-term antibody response to P protein in MRL/LPR mouse "v4".

Fig. 5 is a graph showing the long-term antibody response to P protein in MRL/LPR mouse "v2".

Figs. 6 (a-d) are graphs showing the longitudinal antibody response to ribonucleoproteins P and Sm in 4 control (i.e., untreated) MRL/LPR mice.

Fig. 7 is graph showing results of an ELISA in which the detecting antibody is anti-beef insulin antibody.

Structure

The conjugates of the invention have the general structure recited in the Summary of the Invention above. Examples of preferred structures are those referred to as preferred embodiments herein.

Specific conjugates were made as follows.

Covalent Linkage

Tolerogenic antigen/immunoglobulin conjugates of the invention can be made using disuccinimidyl suberate (DSS), or any other suitable homobifunctional or heterobifunctional cross-linking reagent.

Conjugation can be carried out so as to effect any of a range of protein:IgG molar ratios. The IgG molecules can be of any suitable subclass, with IgG2a and IgG1 subclasses being most preferred for murine IgG because both subclasses bind to Fc receptors on monocytes and lymphoid cells.

DSS, a preferred cross linker of the invention, is a non-cleavable amine-reactive, homobifunctional crosslinking reagent, available from Pierce, Rockford, Illinois. It has been used to link ricin to other proteins, as described in Montesano et al. (1982) Biochem, Biophys. Res. Comm. 109, 7. In general, crosslinking is carried out under mild basic conditions at pH 8 - 8.5, for a time period of 30 - 120 minutes, in a small reaction volume (1-2 ml), and at a concentration of IgG of about 10 mg/ml. By varying the amounts of the crosslinker and reciprocal ratios of the proteins, the conditions necessary for forming a suitable conjugate from a given antigen can be determined.

As is mentioned above, the protein or fragment or peptide thereof that is conjugated to the carrier IgG can be either the entire protein or an immuno-dominant portion of the protein. (The immuno-dominant region can be determined, for example, using X-ray crystallography to reveal portions of the protein that are external and therefore more susceptible to antibody recognition, or by injecting fragments of the protein into an animal and measuring the resulting immune response.) Specific examples of DSS promoted crosslinking of P protein and Factor VIII Light chain:mouse IgG2a and pidgeon cytochrome C:mouse IgG2a are given below; however, the procedure described can be used to cross link any other protein of interest to IgG with appropriate modification which should be apparent to anyone of ordinary skill in this field.

Factor VIII

The undesirable formation of anti-Factor VIII antibody is a major problem in hemophiliac patients treated with Factor VIII replacement therapy; about 40-60% of these antibodies are directed to the light chain, whereas the remaining antibodies formed are specific for

the heavy chain. A tolerogenic IgG/Factor VIII Light chain (F8L described in Kaufman et al., WO 8704187, hereby incorporated by reference) conjugate was made using DSS as the crosslinking reagent. The 76 kD domain light chain of Factor VIII was linked to affinity purified Balb/c myeloma IgG2a, as follows.

F8L and IgG2a were first mixed together in borate saline, pH 8.3. The DSS reagent was dissolved in DMSO (dimethyl sulfoxide) at a concentration of 20 mg/ml and a desired amount of the F8L/IgG2a/DMSO was then mixed with the antigen/Ig mixture by gentle inversion. The pH was then readjusted to 8.3 with 0.1N or 1N NaOH, and monitored and re-adjusted every 20 min. until it stabilized after approximately 90 - 120 min. The reaction was then quenched with 100 times molar excess of glycine, adjusted to pH 8.3, and the reaction allowed to proceed for an additional 2 hours. The mixture was then centrifuged and the conjugate purified by gel filtration chromatography using Ultragel AcA 44 or Sephacryl S300. The gel chromatography of the F8L-mouse IgG conjugate yielded three different but overlapping fractions with the following molar ratios: A1, 5 IgG/1 F8L; A2, 3 IgG/1 F8L; and A3, 1 IgG/1 F8L.

A 25 mg dose of tolerogen conjugate was administered intraperitoneally within 30 hr. parturition to 3 sets of 5 - 8 Balb/c newborn mice (Jackson Laboratory, Bar Harbor, ME). On days 17 and 24, 2 mice from each group were challenged with F8L in RIBI adjuvant. Serum samples were collected by retroorbital plexus puncture on days 14, 21, and 28. These sera were assessed by ELISA for antibodies directed against the antigen KLH, intact Factor VIII, and F8L. It was found that specific non-responsiveness to F8L was induced in ≥80% of the animals, and remained demonstrable throughout

the 28 day experimental period, regardless of the IgG/F8L molar ratio of the tolerogenic conjugate injected. Each of fractions A1 - A3 was capable of inducing neonatal tolerance against the tolerogen, but none rendered the mice tolerant to intact Factor VIII.

In adult animals, in contrast to newborns, the molar ratio of the conjugate was found to be important. In animals with an ongoing immune response to both Factor VIII and the light chain, the results were as follows. In two mice treated with a single injection (25 mg) of conjugate A2 (IgG/F8L, 3:2), there was a significant drop of antibody to Factor VIII light chain (Fig. 1). (In Fig. 1, open and solid triangles indicate two different mice treated with tolerogen on day 0; the open circles represent a mouse treated with IgG as a control; and the closed circles represent a mouse treated with unconjugated F8L as a control. Antibodies to F8L were quantitated on designated days after treatment by injection using ELISA.) In contrast to specific suppression of F8L antibodies in mice treated with the A2 tolerogen, mice treated with a different molar ratio (A1, 5:1 or A3, 1:1) of IG/F8L conjugate made antibody to F8L. This experiment was repeated in 10 Balb/c mice preimmunized with Factor VIII, and antibody to F8L was detected in all 10 mice. Six mice with titer of greater than 1000 were treated by a single intravenous injection of 50 mg of Ig/F8L conjugate A2 (molar ratio of 3:2) and serum samples were collected 7 and 14 days following administration of the tolerogenic conjugate or control substance. Fig. 2 (right panel) shows titers of anti-F8L antibody for each of the six mice tested; in 5 out of 6 mice, there was a significant reduction of anti-F8L antibody; in 2 out of the 5 tolerized mice, there was a total disappearance of F8L antibody titer, and in the 3 other mice, there was a

transient reduction of F8L antibody. Fig. 2 (left panel) shows that none of the 4 control mice treated with either light chain alone (closed circles) or IgG alone (open circles) showed a significant reduction of anti-F8L antibody. In all mice tested, the antibody response to intact Factor VIII was unaffected.

The above experiments demonstrate that antibody to the light chain of Factor VIII is specifically suppressed in newborn mice regardless of the molar ratio of the conjugate; in adult animals with an ongoing immune response, antibody to F8L was also suppressed by the A2 conjugate. In both newborn and adult mice, tolerance appears to be directed specifically to F8L, and does not extend to intact Factor VIII.

Cytochrome-IgG

Cytochrome is not associated with an immune disease, but does induce an immune response in mice *in vivo*, manifested by T cell proliferation. This immune system thus provides a model for T cell non-responsiveness *in vivo* and also permits study of the parameters of crosslinking for the tolerogen conjugate; i.e., the cytochrome causative protein is well-characterized, and the molar ratio of cytochrome/IgG is easy to control.

To increase the ratio of cytochrome to IgG in the conjugate, the amounts of cytochrome and IgG in the reaction mixture are kept identical, e.g., 0.6 mg and 0.9 mg, respectively, while the amount of the bifunctional cross linking reagent is increased, e.g., in steps of 1.2 mg. This results in a higher substitution in the presumed monomer peak, and may also cause increase the degree of polymerization of molecules within the conjugate.

The following experiment was designed to prevent a T cell proliferation response *in vitro* rather than to suppress the ongoing response. A tolerogenic conjugate of

pidgeon cytochrome C (Sigma Chemical Co., St. Louis, MO) and mouse IgG2a (0.41 mole/1 mole) was made and purified as described above for Factor VIII Light chain, using DSS as the crosslinking reagent.

B10.A mice (Jackson Labs) were immunized with 20 nmoles of pidgeon cytochrome C emulsified in Freund's complete adjuvant containing Mycobacterium tuberculosis, (strain H37Ra, Difco Laboratories, Detroit, MI). A total volume of 0.2 ml was distributed in the hind (0.05 ml) and front (0.03 ml) footpads and at the base of the tail (0.04 ml). On day 0, the conjugate or control substance or test substance (see below) was administered to B10.A mice (Jackson Lab); on day 4, the mice were immunized with pidgeon cytochrome C (20 nmoles); and on day 11, the draining lymph nodes (auxillary, inguinal, and paraaortic) were removed and cultured in vitro.

Single cell suspensions of lymph tissue were prepared in Click's (EHAA) medium (BioFluid Inc., Rockville, MD) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), and the T cells isolated by passage over nylon wool. 4×10^5 nylon-adherent cells were cultured in flat bottom 96 well culture plates (Costar #3596, Cambridge, MA) along with 1×10^5 irradiated (3,300R) B10.A spleen cells as a source of antigen-presenting cells, and various concentrations of antigen in a total volume of 0.2 ml of EHAA medium. Cultures were incubated at 37°C in a humidified, 3% CO_2 atmosphere for 4 days. One mCi of ^{3}H-methyl-thymidine, specific activity 6.7 Ci/mmole (New England Nuclear, Boston, MA) was added 16-18 hr. before harvesting the culture. The cells were collected onto glass fiber filter papers using a semiautomated harvesting device (PhD Harvester, Cambridge, MA), lysed with distilled water, and the incorporation of thymidine into DNA measured by

standard liquid scintillation counting techniques.

T cell proliferation was found to be proportionate to incorporation of radioactive thymidine into cellular DNA. Fig. 3 (left panel) shows that administration of the pidgeon cytochrome C/IgG2a tolerogenic conjugate (PC-RPC5, 0.41 mole/mole, open circles) resulted in almost complete suppresion of T cell proliferation. In contrast, the untreated control (closed circles) showed a maximal T cell proliferation response of 60,000 cpm. This figure also shows the results of the proliferative response after administration of IgG2a alone (RPC5, a Balb/c myeloma protein, open squares), a horse cytochrome C/IgG2a conjugate (HC-RPC5, substituted 0.41 mole/mole, open triangles), an unconjugated mixture of pidgeon cytochrome C and IgG2a (PC + RPC5, 0.41 mole/mole, upside down open triangles), pidgeon cytochrome C alone (PC, closed squares), and pidgeon cytochrome C conjugated with splenocytes (PC.501, Jenkins et al., 1987, J. Expt. Med. 165:302). Fig. 3 (middle panel) shows the results of the same experiment using smaller doses of the pidgeon cytochrome C challenge antigen, and (right panel) the proliferative response after challenge with the control antigen PPD, which is found in Freund's adjuvant. Although the latter conjugate suppressed the T cell proliferative response significantly, the response was an order of magnitude greater than the tolerogenic conjugate of the invention. Pidgeon cytochrome C suppressed the proliferative response by approximately 50%, but horse cytochrome C, which differs from pidgeon cytochrome C by only one amino acid, suppressed the response by only about 25%, indicating that tolerance induction by the pidgeon cytochrome C/IgG2a conjugate was highly specific.

The above data suggest that the construction of a tolerogenic conjugate can also offer a specific

immunotherapy for the treatment of unwanted immune responses which originate in T cell responsiveness.

P Protein

P protein is a ribonucleoprotein of 2500 molecular weight (obtained from Cornell Univ. and Hoffman LaRoche). Patients with the central nervous system manifestation of SLE are known to make antibodies to P protein. A useful model system for treatment is MRL mice, which at 4 - 5 months of age spontaneously form antibodies to P protein.

A tolerogen conjugate was formed as above by linking P protein to mouse IgG2a. Figs. 4 and 5 are graphs representing the antibody response to P protein in two mice following intravenous administration of three small doses (18.5 mg, 37 mg, and 55.5 mg) of the tolerogenic conjugate. In both mice, the long-term anti-P protein antibody response was suppressed after treatment with the tolerogenic conjugate. However, the short-term anti-P response varied; mouse "v2" showed a slight increase in anti-P antibody, followed by a precipitous decrease after approximately 6 weeks, whereas mouse "v4" showed a gradual increase in anti-P antibody immediately following administration of the conjugate, followed by a gradual decrease after approximately 8 weeks.

Fig. 6 is a comparison of the antibody responses to ribonucleoproteins P and Sm in four untreated MRL/LPR mice. Although the four mice, #92, #97, #101, and #104, exhibited varying antibody titers for both specificities, indicating that these antibody responses undergo spontaneous fluctuation, at 5 - 6 months of age, the level of anti-P protein antibody is elevated in all control untreated mice.

Myasthenia Gravis

Myasthenia gravis (MG) is an autoimmune disease

caused at least in part by an immune response to a natural component of the body, the acetyl choline receptor (ACR), which may prevent transmission of nerve impulses by blocking transmission of neurotransmittors across the synapse. Symptoms of the disease are tissue damage, muscle weakness, and, in certain cases, death.

It is possible to cause an immune response to the acetyl choline receptor in mice by immunization with the receptor. ACR is a heterodimeric protein composed of a and ß subunits. The a chain has several immunodominant regions; one region in particular, amino acids 107-204, may be especially effective when used as a tolerogenic conjugate in the mouse model system. A tolerogenic conjugate consisting of the aARC peptide, corresponding to amino acids 172-204 of the a chain, linked to Balb/c myeloma IgG2a (RPC5) can be administered to mice, causing the mice to become tolerant to aACR.

Pemphigus Vulgaris

Pemphigus Vulgaris (PV) is a blistering autoimmune disease affecting the skin and mucous membranes. When left untreated, or when inappropriately treated, it is fatal. PV is currently treated with high doses of systemic steroids and immunosuppressive agents. The hallmark of the disease is the production an antibody against the intercellular cement sustance (ICS) of the epidermis. The antibody is deposited in vitro and can be demonstrated in the sera of patients. The pathogenetic role of the antibody has been demonstrated in studies of infants born to mothers with pemphigus vulgaris, who develop the disease by the transplacental transfer of the antibody.

The most significant observation in the study of the molecular mechanisms of pathogenesis have come from the mouse model of pemphigus. In this model, high titer

human sera from PV patients, when injected intraperitoneally into Balb/c mice, produced clinical histological and immunopathological PV. The IgG fraction of human sera causes mouse PV, which can be reversed or inhibited by pretreatment of the mice with high doses of systemic corticosteroids.

The protein which is believed to be the causative protein of PV has been biochemically characterized, although the results have been conflicting because of differences in substrates and techniques of extraction.

Studies using epidermal extract and keratinocyte cultures employing immunoblotting and immunoprecipitating techniques indicate that the PV antigens are 160 or 140 kD glycoproteins. Studies using tumor cells derived from squamous cell carcinoma of the skin indicate that the PV antigen is 110 kD. Using a sensitive RIA, it has been demonstrated that the majority of PV sera bind to these cells.

Another model system for PV is an in vitro system in which human lymphoid cells, e.g., peripheral blood leukocytes (PBL) from PV patients, produce antibodies against the PV antigen. Cells from normal patients or patients with other bullous diseases do not produce anti-PV antibodies. The in vitro synthesized antibody can be easily assayed. In addition, unfractioned bone marrow cells from patients with active disease also produce the antibody in vitro.

The PV protein can be isolated from tumor cells derived from patients with squamous cell carcinoma, according to conventional methods. Serum antibodies from 15 PV patients have been known to recognize a 110 kD protein on the surface of such cells, which can be cultured to provide a source for the causative 110 kD protein. The protein will be isolated using high titer PV

sera mouse monoclonal antibody to PV antigen.

Once the PV protein is available, its efficacy in restoring tolerance will be tested in the mouse model. Balb/C mice will be injected with PV antigen linked to IgG and then injected with PV sera to determine if the clinical disease can be prevented. Similarly, PBL from patients with active disease will be exposed to PV antigen and then studied for their ability to produce PV autoantibodies.

Should the mouse model indicate that the disease in the mouse can be prevented, or the autoantibody production in vitro reduced or abolished, attempts will be made to isolate the relevant epitope by serial fragmentation of the PV antigen and to extend these studies to the human level.

Autoantibody to Insulin

An autoimmune disease in which the immune system recognizes insulin as a foreign antigen may be treated by tolerizing the immune system to a conjugate containing the insulin protein or a fragment of insulin. The following experiments demonstrate that a conjugate of beef insulin fragments coupled to mouse IgG tolerizes mice to additional exposure to beef insulin.

The tolerogenic conjugate was made by linking beef insulin proteolytic fragments to mouse IgG2a using DSS, as described above. After fractionation by gel filtration on Sephacryl S300 Superfine, three fractions (Peaks I, II, and III) were obtained. The tolerogenicity of each fraction was tested in Balb/c mice which had been immunized with whole beef insulin. Both T and B cell responses to the insulin-IgG conjugates were tested.

The B cell response to the insulin-IgG conjugates was measured by the ability of insulin-immunized mice to produce antibodies specific for beef insulin. Immunized

Balb/c mice were tolerized with 0.2 mg/ml of conjugates. Eight days later, mice were challenged with 25 ug beef insulin in complete Freund's adjuvant (CFA) and boosted with the same antigen in incomplete Freund's adjuvant (IFA). Mice were bled 10 days later and serum anti-beef insulin antibody measured by ELISA. The results, presented in Fig. 7, represent anti-beef insulin antibody binding minus the background binding of antibodies present in normal serum.

The T cell response to the insulin-IgG conjugate was measured using a T cell proliferation assay, as described above for the pidgeon Cytochrome C-IgG conjugate. On day 0, mice were injected with 0.2 mg each of insulin conjugated with mouse IgG (RPC-5). On day seven, mice were challenged with 25 ug of beef insulin/CFA. On day 15, lymph nodes were collected and 10^6 cells/well were incubated in triplicate in complete medium with antigen. On day 19, cells were pulsed with 1 uCi/well of ^{3}H-Tdr; incorporation was measured 24 hours later.

The results of B cell and T cell assays, presented in Fig. 7 and Table 1, respectively, show that both B and T cell immune responses were suppressed by the three tolerogenic conjugates (Sephacryl fractions 51-68 or Peak I, 69-85 or Peak II, and 86-97 or Peak III) compared to mice injected with gamma globulin alone.

Any autoimmune disease in which insulin is recognized as a foreign antigen, e.g. insulin-dependent "juvenile" diabetes, may be treated according to the invention, using tolerogenic conjugates of human insulin or fragments thereof coupled to human IgG.

Use

The conjugates of the invention can be

administered using conventional techniques, e.g., intravenous administration. Amounts and frequency will vary with application; generally, administration will need to be repeated at intervals, e.g., weekly, and may need to be more frequent during periods of disease flare-up. Dosages will generally be in the range of those used for oligonucleotide conjugates, Borel et al., U.S.P. 4,650,675, supra, generally be in the range of about 1 to 250 mg/kg/day.

Mechanism of Action

The precise mechanism of action by which protein or protein fragment-immunoglobulin conjugates of the invention can induce tolerance to the causative protein is not known. Tolerogens of the invention can affect both T and B cell immune responses, suggesting that both cellular and humoral immunity can be specifically suppressed by tolerogen conjugate administration.

B cell tolerance is a positive event that requires protein synthesis. The mechanism for tolerance in B cells, or B cell non-responsiveness to a given protein, may occur by a mechanism of receptor blockade. The Fc receptor for the IgG may help focus the antigenic determinant which actively mediates a tolerogenic signal (Waldsmidt et al., J. Immunol. 131:2204 (1983)), and thus create a double binding on the same B cells. For example, in the disease MG, the immunoglobulin receptor on B cells that produce an antibody specific for the acetylcholine receptor may be cross-linked by the Fc portion of the IgG molecule of the conjugate and the acetylcholine receptor (or fragment) portion of the conjugate. This dual crosslink may prolong the time of contact between the conjugate and the B cell, enabling the conjugate to remain on the cell surface for the time which is necessary to signal the cell to stop producing antibodies that are

specific for the acetylcholine receptor. After the tolerogen has been removed from the cell surface either by tissue culture <u>in vitro</u> or by pronase treatment, the B cell remains tolerant (see Aldo-Benson et al., J. Immunol. 114:141 (1975); Aldo-Benson et al., Cell. Immunol. 71:99 (1982)). Thus suppression may occur at a critical point in the cell cycle (e.g., before or during DNA synthesis), or prior to T cell factor influence.

Natural tolerance originates in the thymus. In addition, thymectomy is known to result in autoimmune phenomena (Yunis, E.J., J. Exp. Med. 125:947 (1967); Wick et al., J. Immunol. 104:54 (1970)). Tolerance in T cells, or T cell non-responsiveness to a given protein, may occur by clonal deletion, immunoregulation by suppressor T cells, or direct inactivation of T cells by the protein. Clonal deletion has been demonstrated for self MHC (Kappler et al., Cell 49:273 (1987)) and extended to non MHC antigen (Kappler et al., Nature 332:35 (1988); MacDonald et al., Nature 332:40 (1988)), and may also be the mechanism for tolerance to class I alloantigens on cell surfaces (Kappler et al., Cell 49:273 (1987)). Whether clonal deletion is also applicable to autologous soluble protein antigen presented by class II MHC molecules is unknown, as is the role of the T cell receptor. The possibility that helper T cells are down-regulated by suppressor T cells could be a fail-safe mechanism to protect the host against autoimmunity. Thus, T cell tolerance could be explained by functional T cell inactivation by tolerogen, without physical deletion. Consistent with this interpretation are studies demonstrating showing that two different epitopes on the same antigen mediate either clonal inactivation or suppression (Shastri et al., J. Exp. Med. 162:332 (1985)). In addition, it has been shown that specificity

of the epitope which is responsible for either T cell proliferation or direct inactivation of the T cell clone is identical (Jenkins et al., J. Exp. Med. 165:302 (1987)).

Hapten conjugated IgG induces tolerance in T cells of mice and humans (Borel et al., J. Exp. Med. 142:1254 (1975); Geha et al., J. Clin. Invest. 65:1509 (1980); Morimoto et al., Cell. Immunol. 82:415 (1983)). Antigen presenting cells together with class II MHC may present the tolerogen to the T cell receptor, but whether both CD4 and CD8 T cells are rendered unresponsive is unkonwn. Prostaglandin (PG2) favors tolerance induction (Goldings, J. Immunol. 136:817 (1986)), whereas interleukin 2 (IL2) favors immunogenicity and may prevent or reverse tolerance (Sadegh-Nasseri et al., Eur. J. Immunol. 18:417 (1988)). Thus, tolerance in T cells may occur through the production of PG2-like lymphokines. The tolerogen, in this case the conjugate, may stimulate the T helper cell to produce PG2, and thus prevent T helper cell function in B cell antibody production.

Other Embodiments

Other embodiments are within the following claims. For example, tolerogenic conjugates of the invention may be used to treat any disease in which an immune response to a protein causes unwanted symptoms. Thus conjugates of the invention may be used to treat autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, or infertility resulting from autoantibodies, e.g., to human leutinizing hormone receptor in women. Tolerogenic conjugates may also be used to treat congenital diseases resulting in unwanted immune responses; e.g., congenital lupus, in which the fetus experiences heartblock because of transplacental transfer of maternal antibodies specific for ribonucleoprotein La; or coelic disease, in which a genetically determined

intolerance to gliadin, a component of wheat results in the inability to digest wheat. In addition, allergies caused by an unwanted immune response to a foreign protein may be treated according to the invention; e.g., certain food or plant allergies. Tolerogenic conjugates may also be used to suppress unwanted immune responses to therapeutic human natural or monoclonal antibodies; e.g., patients receiving the therapeutic monoclonal antibody OKT3, which is used to treat cancer and may require repeated doses of the antibody, sometimes is associated with an unwanted immune response; administration of a tolerogenic conjugate consisting of the OKT3 antibody crosslinked to an isologous human IgG carrier may prevent an immune response to antibodies used in diagnostic targeting, e.g., to image tumors or deliver drugs.

Tolerogens can be made using any soluble, isologous immunoglobulin, e.g., human IgG or IgA. IgG is preferred because the Fc fragment participates in the immunological functioning of the conjugates. The conjugates may be administered parentally, in the case of IgG, or enterally, in the case of IgA, which is more resistant to digestion than IgG. Oral tolerance to food allergens, e.g., bovine ß-lactoglobulin, or immunogens, may be induced by linking these antigenic determinants to IgA.

In addition, proteins may be linked to the immunoglobulin carrier without aggregation of the conjugates and fetal autoimmune disease can be treated because of the ability of IgG to cross the placenta.

CLAIMS

1. A tolerogenic conjugate for inhibiting an immune response in a mammal to a protein, said conjugate comprising said protein, or a fragment or peptide thereof, covalently linked to a soluble isologous immunoglobulin carrier molecule.

2. The conjugate of claim 1 wherein said protein is an isologous protein and said immune response is characteristic of an autoimmune disease.

3. The conjugate of claim 1 wherein said immunoglobulin is IgG.

4. The conjugate of claim 1 wherein said fragment or peptide is, when not linked to said immunoglobulin molecule, immunogenic in said mammal.

5. A method of inhibiting, in a mammal, an immune response to a protein, said method comprising administering to said mammal an immune response inhibiting amount of a tolerogenic conjugate comprising said protein, or a fragment or peptide thereof, covalently linked to an isologous immunoglobulin molecule.

6. The method of claim 5, wherein said immunoglobulin is IgG.

7. The method of claim 6, said protein comprising P protein and said autoimmune disease being SLE.

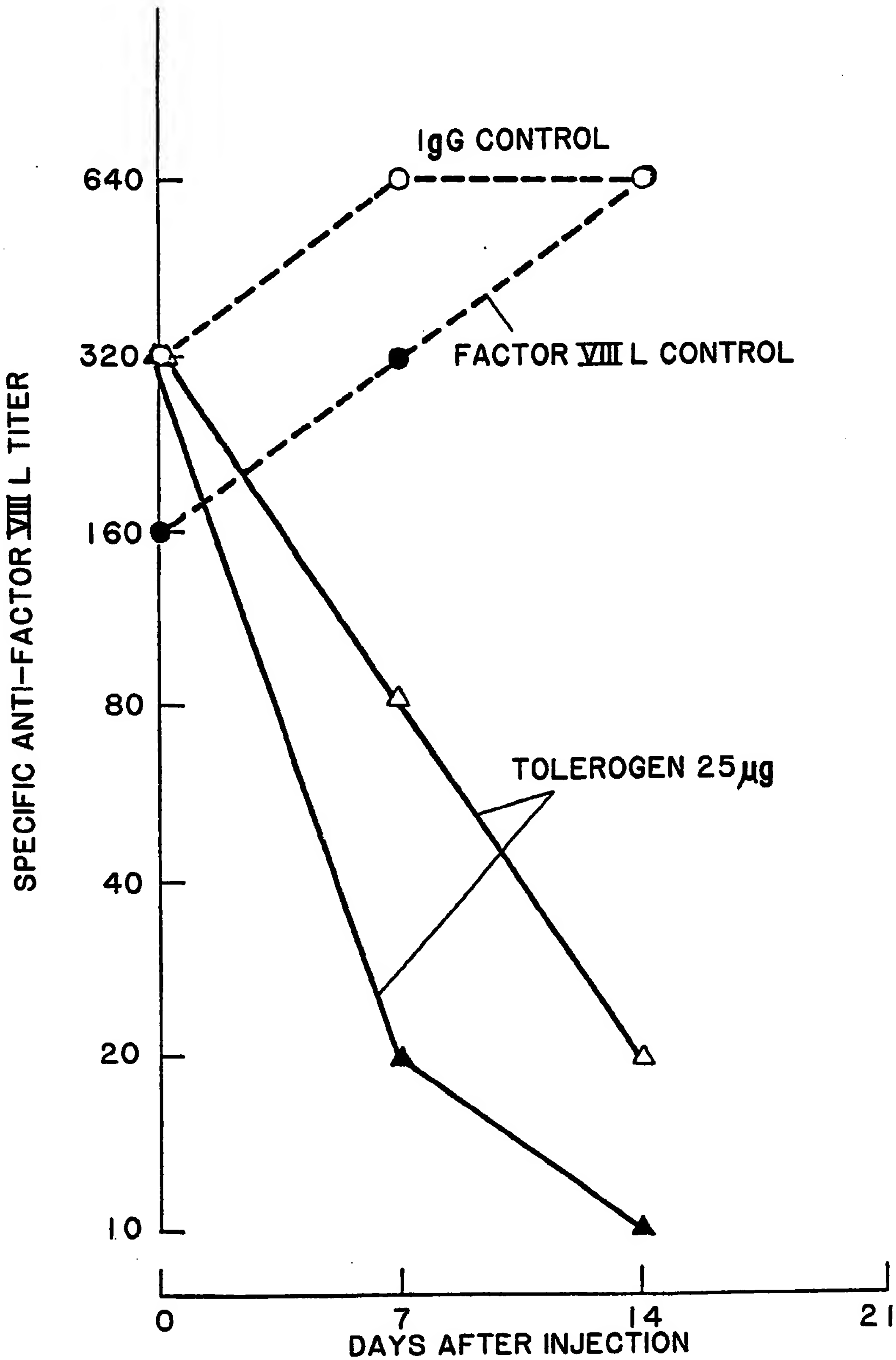
8. The method of claim 6, said protein comprising Factor VIII and said autoimmune disease being an unwanted immune response to isologous Factor VIII.

9. The method of claim 6, said protein being a PV-associated protein and said autoimmune disease being Pemphigus vulgaris.

10. The method of claim 5, said immune response being an allergic response.

11. The method of claim 6, said protein comprising insulin, or a fragment thereof, and said autoimmune disease being insulin-dependent diabetes.

FIG. I


IgG CONTROL
FACTOR VIII L CONTROL
TOLEROGEN 25μg
SPECIFIC ANTI-FACTOR VIII L TITER
640
320
160
80
40
20
10
0
7
14
21
DAYS AFTER INJECTION

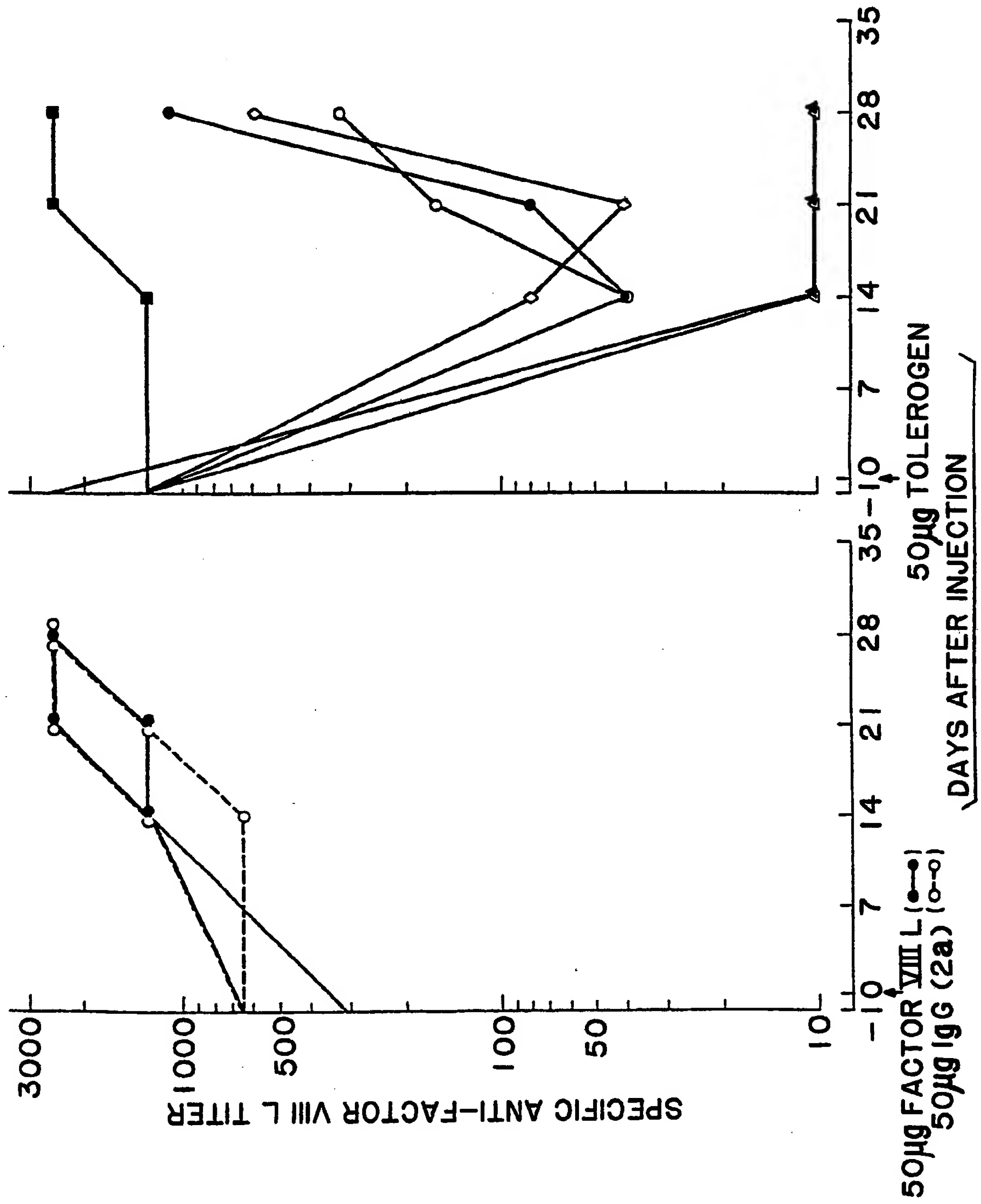

SPECIFIC ANTI-FACTOR VIII L TITER
3000
1000
500
100
50
10
-10
7
14
21
28
35
50µg FACTOR VIII L (●—●)
50µg IgG (2a) (○--○)
35-10
50µg TOLEROGEN
DAYS AFTER INJECTION

FIG. 2

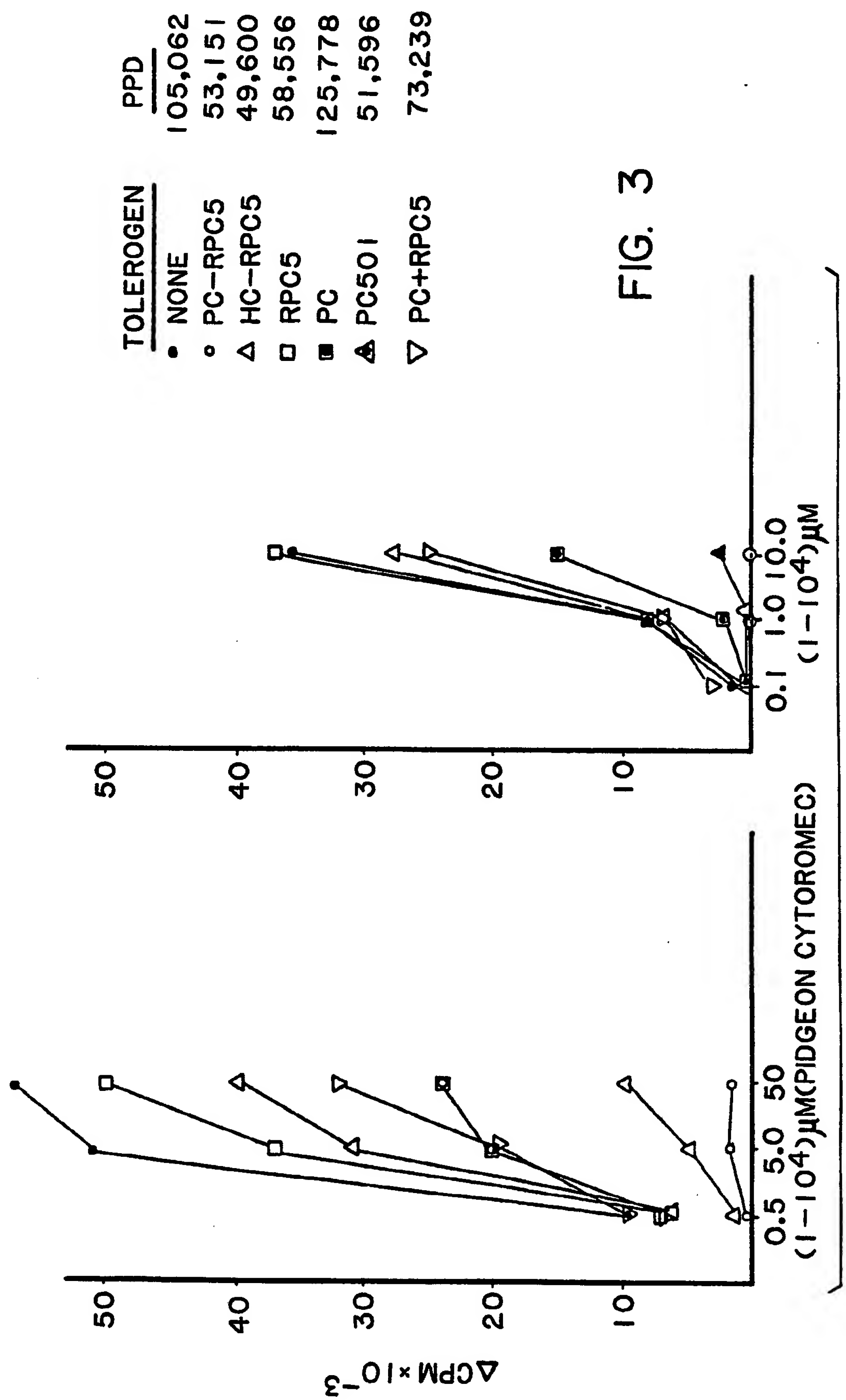

TOLEROGEN
PPD
• NONE 105,062
○ PC–RPC5 53,151
△ HC–RPC5 49,600
□ RPC5 58,556
■ PC 125,778
▲ PC501 51,596
▽ PC+RPC5 73,239
ΔCPM × 10⁻³
50
40
30
20
10
0.5
5.0
50
(1–10⁴)μM(PIDGEON CYTOROMEC)
0.1
1.0
10.0
(1–10⁴)μM

FIG. 3

FIG. 4

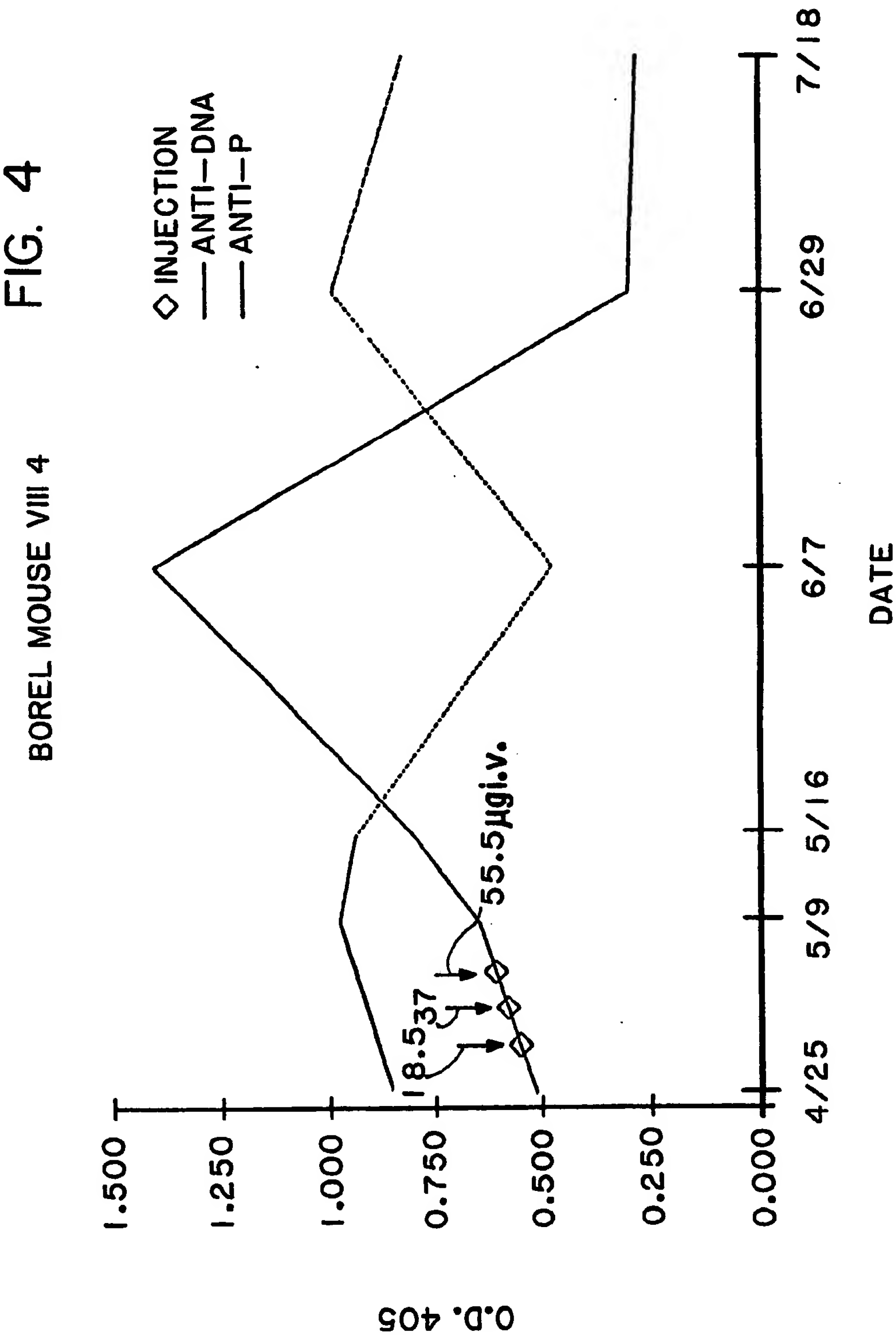

BOREL MOUSE VIII 4
◇ INJECTION
— ANTI—DNA
— ANTI—P
18.5
37
55.5μgi.v.
1.500
1.250
1.000
0.750
0.500
0.250
0.000
O.D. 405
4/25
5/9
5/16
6/7
6/29
7/18
DATE

FIG. 5

BOREL MOUSE V2

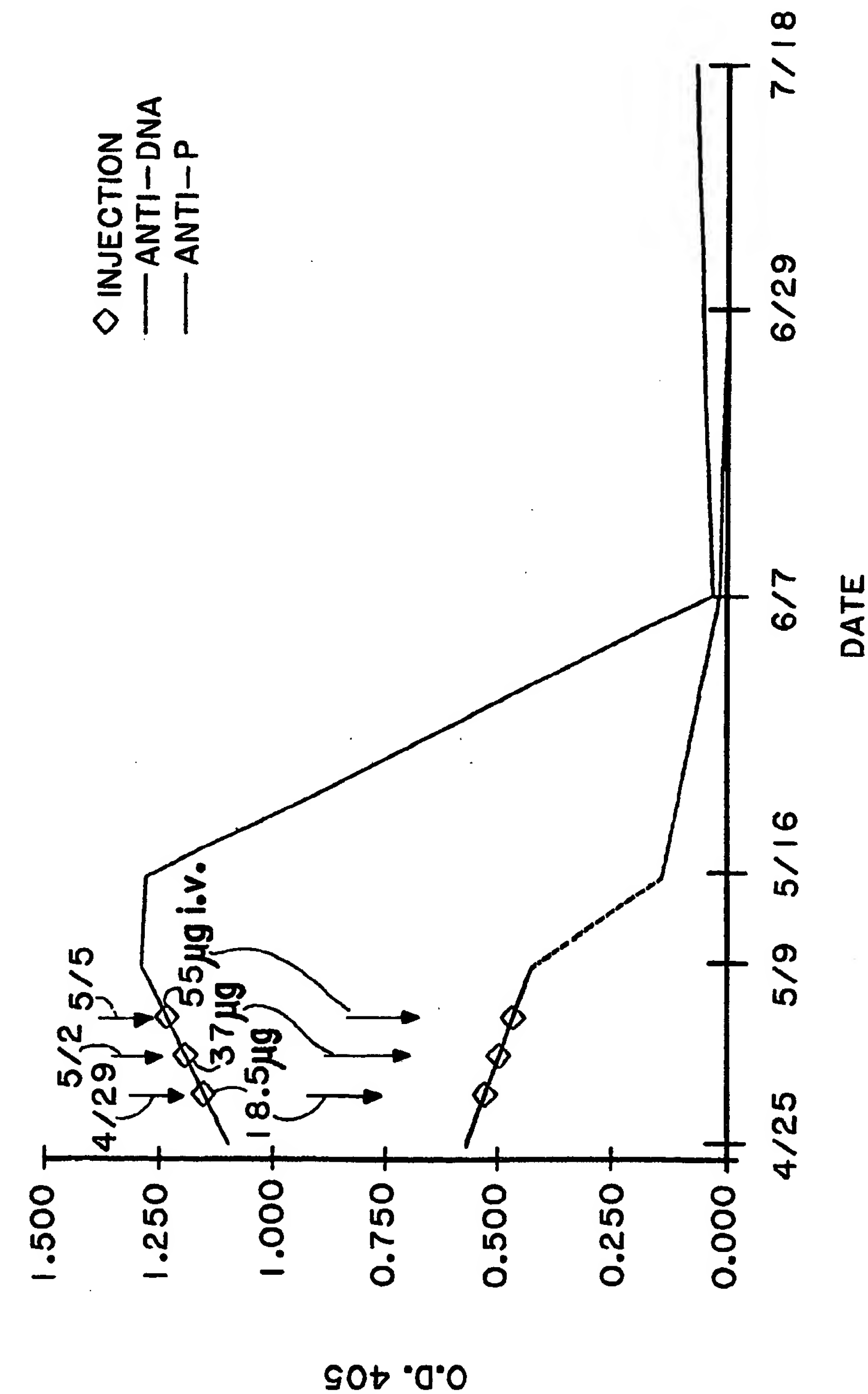
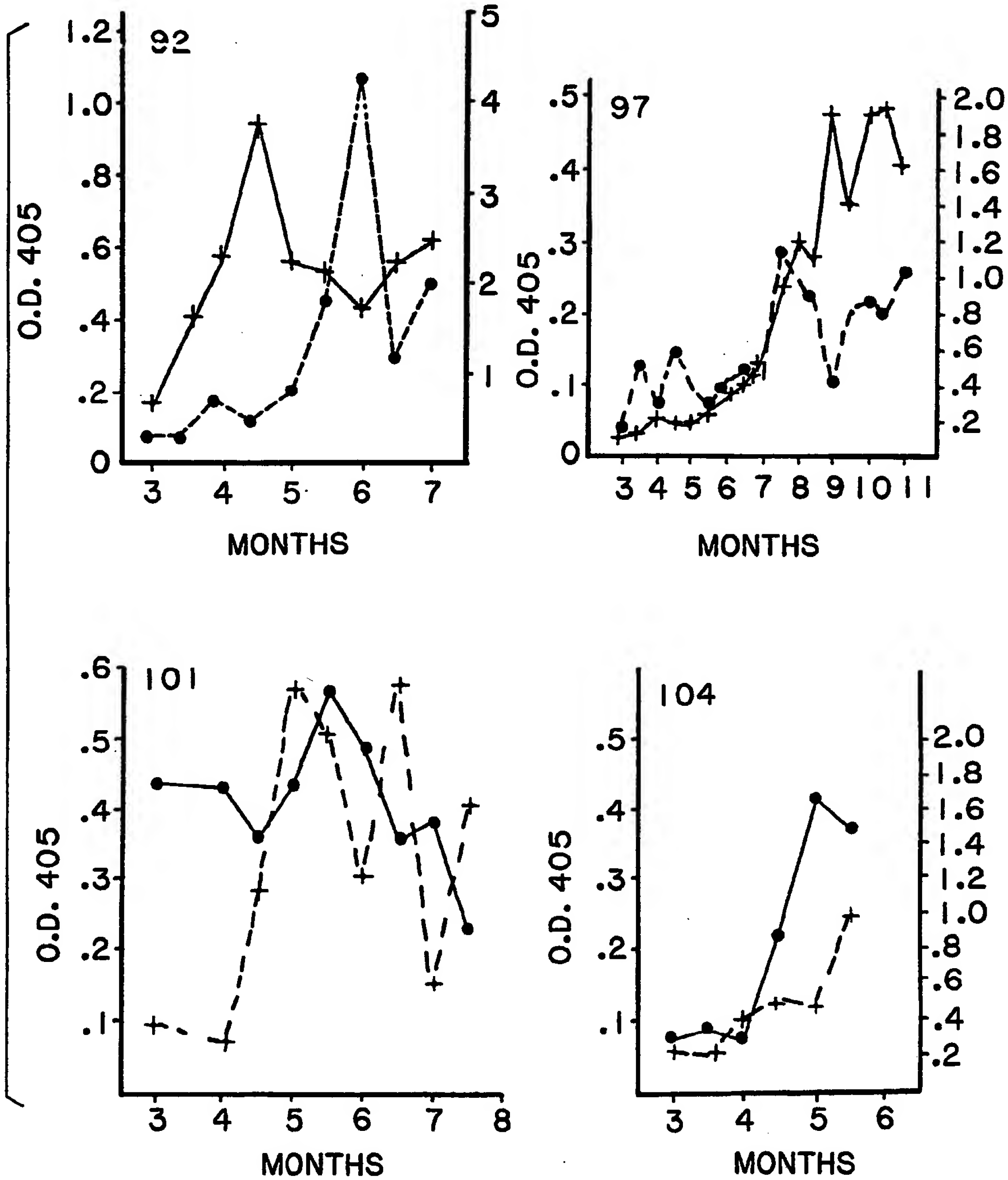


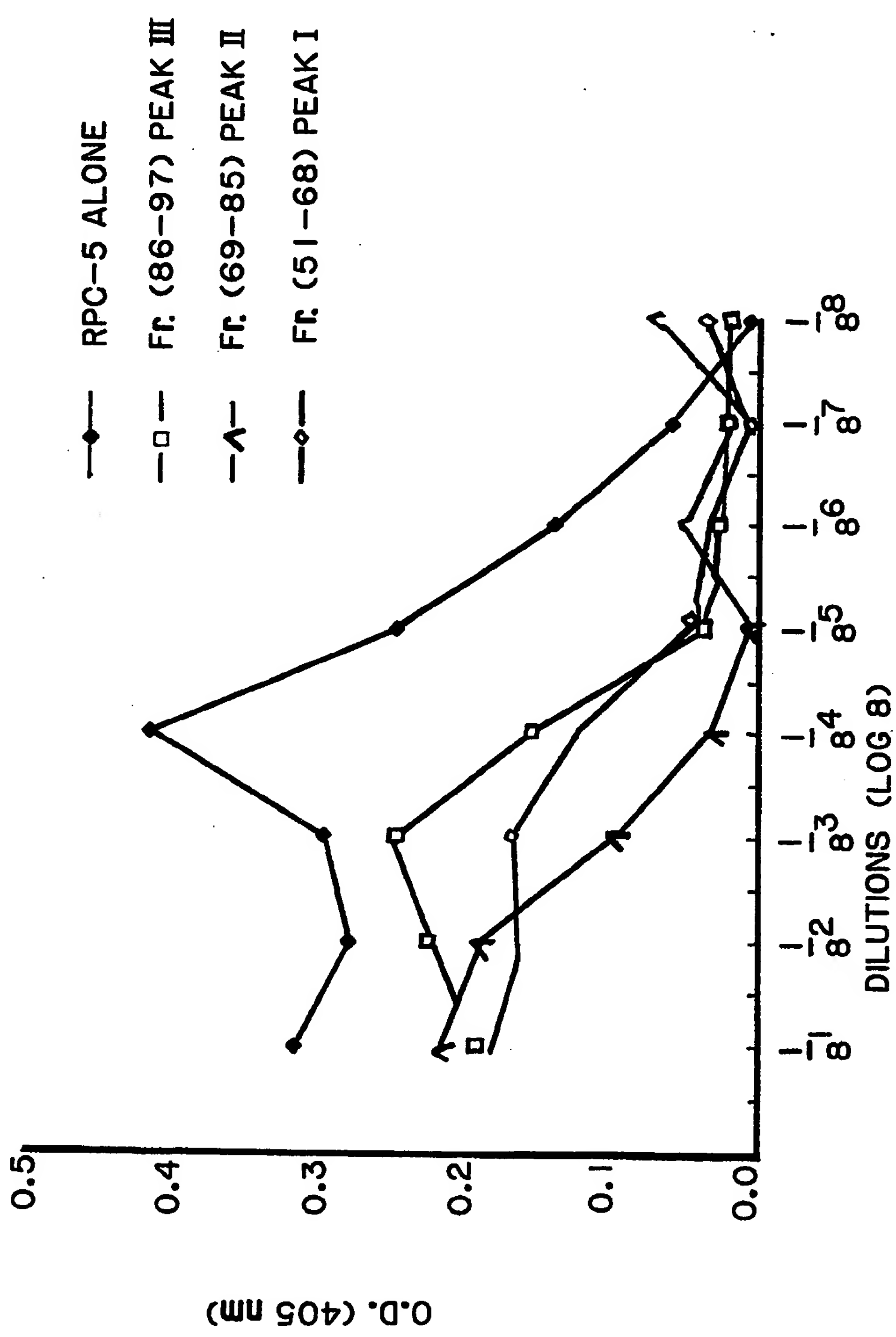
FIG. 6



LONGITUDINAL STUDIES OF ANTI-SM (+---+) AND ANTI-P (●---●) LEVELS IN 4 MRL/LPR MICE POSITIVE FOR BOTH ANTIBODY SPECIFICITIES. THE MOUSE NUMBER IS INDICATED ON THE UPPER LEFT.

FIG. 7

BEEF INSULIN FRAGMENTS–ELISA



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05539

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395

U.S. Cl.: 424/85.8; 530/387,389,391

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85.8; 530/387,389,391

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases; Chemical Abstract Services Online (File CA, 1967-1990); File Biosis, 1969-1990). Automated Patent System (OSPAT) 1975-1990.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,650,675 (BOREL et al.) 17 MARCH 1987 see entire article	1-11
Y	Journal of Immunology, Volume 136 No. 1 01 JANUARY 1986, (Kawamura et al.) "Enhancement of antigenic potency invitro and immumogenicity in vivo by coupling the antigen to anti-immunoglobulin", pp. 58-65 see abstract.	1-11
Y	Nature, Vol. 327 07 MAY 1987, (Carayanniotis et al.), "Adjuvant-free IgG responses induced with antgen coupled to antibodies against class II MHC", pp. 59-61, see abstract.	1-11
Y	Nature, Vol. 261, 06 MAY 1976, (Borel et al.), "Isologous IgG-induced tolerance to benzyl penicilloyl", see pp. 49-50.	1-6, 10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 MAY 1990	22 AUG 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Denise Bernstein for Denise Bernstein

Form PCT/ISA/210 (second sheet) (Rev.11-87)

cdb:6/14/90

International Application No. PCT/US89/05539

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,388,441, (Katz) 14 JUNE 1983 see entire article	1-11
Y	Annals of Allergy, Vol. 50, 1983 (Patel et al.), "Bullous pemphigoid and pemphigus vulgaris", see pp. 146-147	1-6, 9
Y	The Journal of Immunology, Vol. 137, No. 4, 15 AUGUST 1986, (Gutstein et al.), Induction of immune tolerance by administration of monoclonal antibody to L3T4", see pp. 1127 and 1131.	1-11
Y	EP,A, 0117114, (Reinherz et al.) 29 AUGUST 1984, see pages 1-6.	1-11
Y	Basic and Clinical Immunology, 5th edition, 1984, Theofilopoulous Chapter 12, "Autoimmunity", see page 153	1-7, 9-11
Y	Basic and Clinical Immunology, 5th Edition, 1984, (Wells), Chapter 24, "Hemotologic Diseases", see p. 484.	1-6, 8